

BROAD SPECTRUM ANTI-VIRAL THERAPEUTICS AND PROPHYLAXIS

CROSS-REFERENCE TO RELATED APPLICATIONS

5 This application claims benefit of priority to United States Provisional Application Number 60/428,535, filed November 22, 2002, entitled "Broad spectrum anti-viral therapeutics and prophylaxis", herein incorporated by reference, and to United States Provisional Application Number 60/464,217, filed April 19, 2003, entitled "Class of broad spectrum anti-viral protein", herein incorporated by reference.

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BACKGROUND OF THE INVENTION

Field of the Invention

15 The invention relates to therapeutic compositions that can be used to prevent and treat infection of human and animal subjects by a pathogen, and specifically to protein-based therapeutic compositions that can be used for the prevention and treatment of viral infections, such as the prevention and treatment of influenza infection.

Description of Related Art

20 Influenza is a highly infectious acute respiratory disease that has plagued the human race since ancient times. It is characterized by recurrent annual epidemics and periodic major worldwide pandemics. Because of the high disease-related morbidity and mortality, direct and indirect social economic impacts of influenza are enormous. Yearly
25 epidemics cause approximately 300,000 hospitalizations and 25,000 deaths in the United States alone. Four pandemics occurred in the last century; together they caused tens of millions of deaths. Mathematical models based on earlier pandemic experiences have estimated that 89,000-207,000 deaths, 18-42 million outpatient visits and 20-47 million additional illnesses will occur during the next pandemic (Meltzer, MI, Cox, NJ and
30 Fukuda, K. (1999) *Emerg Infect Dis* 5:659-671).

Influenza is typically caused by infection of two types of viruses, *Influenza virus A* and *Influenza virus B* (the third type *Influenza virus C* only causes minor common cold like symptoms). They belong to the *orthomyxoviridae* family of RNA viruses. Both type A and type B viruses have 8 segmented negative-strand RNA genomes enclosed in a lipid envelope derived from the host cell. The viral envelope is covered with spikes that are composed of three types of proteins: hemagglutinin (HA) which attaches virus to host cell receptors and mediates fusion of viral and cellular membranes; neuraminidase (NA) which facilitates the release of the new viruses from host cells; and a small number of M2 proteins which serve as ion channels.

Infections by influenza type A and B viruses are typically initiated at the mucosal surface of the upper respiratory tract. Viral replication is primarily limited to the upper respiratory tract but can extend to the lower respiratory tract and cause bronchopneumonia that can be fatal.

Influenza viral protein hemagglutinin (HA) is the major viral envelope protein. It plays an essential role in viral infection. The importance of HA is evidenced by the fact that it is the major target for protective neutralizing antibodies produced by the host immune response (Hayden, FG. (1996) In *Antiviral drug resistance* (ed. D. D. Richman), pp. 59-77. Chichester, UK: John Wiley & Sons Ltd.). It is now clear that HA has two different functions in viral infection. First, HA is responsible for the attachment of the virus to sialic acid cell receptors. Second, HA mediates viral entry into target cells by triggering fusion of the viral envelope with cellular membranes.

HA is synthesized as a precursor protein, HA0, which is transferred through the Golgi apparatus to the cell surface as a trimeric molecular complex. HA0 is further cleaved to generate the C terminus HA1 (residue 328 of HA0) and the N terminus of HA2. It is generally believed that the cleavage occurs at the cell surface or on released viruses. The cleavage of HA0 into HA1/HA2 is not required for HA binding to sialic acid receptor; however, it is believed to be necessary for viral infectivity (Klenk, HD and Rott, R. (1988) *Adv Vir Res.* 34:247-281; Kido, H, Niwa, Y, Beppu, Y and Towatari, T. (1996) *Advan Enzyme Regul* 36:325-347; Skehel, JJ and Wiley, DC. (2000) *Annu Rev Biochem* 69:531-569; Zambon, M. (2001) *Rev Med Virol* 11:227-241.)

Currently, influenza is controlled by vaccination and anti-viral compounds. Inactivated influenza vaccines are now in worldwide use, especially in high-risk groups. The vaccine viruses are grown in fertile hen's eggs, inactivated by chemical means and purified. The vaccines are usually trivalent, containing representative influenza A viruses (H1N1 and H3N2) and influenza B strains. The vaccine strains need to be regularly updated in order to maintain efficacy; this effort is coordinated by the World Health Organization (WHO). During inter-pandemic periods, it usually takes 8 months before the updated influenza vaccines are ready for the market (Wood, J. (2001) *Phil Trans R Soc Lond B* 356:1953-1960). However, historically, pandemics spread to most continents within 6 months, and future pandemics are expected to spread even faster with increased international travel (Gust, ID, Hampson, AW., and Lavanchy, D. (2001) *Rev Med Virol* 11:59-70). Therefore it is inevitable that an effective vaccine will be unavailable or in very short supply during the first waves of future pandemics.

Anti-viral compounds have become the mainstay for treating inter-pandemic diseases. Currently, they are also the only potential alternative for controlling pandemics during the initial period when vaccines are not available. Two classes of antiviral compounds are currently on the market: the M2 inhibitors, such as amantadine and rimantadine; and the NA inhibitors, which include oseltamivir (Tamiflu) and zanamivir (Relenza). Both classes of molecules have proven efficacy in prevention and treatment of influenza. However, side effects and the risk of generating drug-resistant viruses remain the top two concerns for using them widely as chemoprophylaxis (Hayden, FG. (1996) In *Antiviral drug resistance* (ed. D. D. Richman), pp. 59-77. Chichester, UK: John Wiley & Sons Ltd.). Most importantly, future pandemic strains, either evolved naturally or artificially created by genetic engineering in bio-warfare, may be resistant to all the available anti-viral compounds, and this will have devastating consequences globally.

In summary, currently available vaccination and anti-viral compounds are limited by some fundamental shortcomings. Novel therapeutic and prophylactic modalities are needed to address future influenza pandemics.

BRIEF SUMMARY OF THE INVENTION

The present invention recognizes that current therapeutics for preventing and treating infection by pathogens are often difficult to provide in a timely manner, can have undesirable side effects, and can lead to drug-resistant pathogen strains.

The present invention provides new compositions and methods for preventing and treating pathogen infection. In particular, the present invention provides compounds having an anchoring domain that anchors the compound to the surface of a target cell, and a therapeutic domain that can act extracellularly to prevent infection of the target cell by a pathogen, such as a virus.

In one aspect, the invention provides a protein-based composition for preventing or treating infection by a pathogen. The composition comprises a compound that comprises at least one therapeutic domain comprising a peptide or protein, where the therapeutic domain has at least one extracellular activity that can prevent the infection of a target cell by a pathogen, and at least one anchoring domain that can bind at or near the membrane of a target cell.

In some embodiments of this aspect of the present invention, the at least one therapeutic domain comprises an inhibitory activity that prevents or impedes the infection of a target cell by a pathogen. In a preferred embodiment, the inhibitory activity inhibits the activity of a protease that can process a viral protein necessary for infection of a target cell. In a particularly preferred embodiment, the compound comprises a therapeutic domain that can inhibit the processing of the HA protein of influenza virus, and the anchoring domain can bind the compound at the surface of a respiratory epithelial cell.

In some embodiments of the present invention, at least one therapeutic domain comprises a catalytic activity. In a preferred embodiment, the catalytic activity removes a moiety from the surface of a target cell that is necessary for infection of the target cell. In a particularly preferred embodiment, the therapeutic domain is a sialidase that can digest sialic acid moieties on the surface of epithelial target cells, and the anchoring domain is a GAG-binding domain of a human protein that can bind heparin or heparan sulfate moieties at the surface of an epithelial cell.

In another aspect, the present invention includes a pharmaceutical composition for treating or preventing pathogen infection in a subject. The pharmaceutical composition comprises a compound of the present invention comprising at least one therapeutic domain and at least one anchoring domain. The pharmaceutical composition can also
5 comprise solutions, stabilizers, fillers and the like. In some preferred embodiments, the pharmaceutical composition is formulated as an inhalant. In some preferred embodiments, the pharmaceutical composition is formulated as a nasal spray.

In yet another aspect, the present invention includes a method for treating or preventing infection by a pathogen. The method includes applying a pharmaceutically
10 effective amount of a compound of the present invention to at least one target cell of a subject. Preferably, the pharmaceutical composition is applied by the use of a spray or inhalant.

15 BRIEF DESCRIPTION OF SEVERAL VIEWS OF THE DRAWINGS

Figure 1 is a schematic depiction of the primary amino acid structure of aprotinin.

Figure 2 shows GAG-binding sequences of four human genes: PF4, human platelet
20 factor 4; IL8, human interleukin 8; AT III, human antithrombin III; ApoE, human apolipoprotein E; AAMP, human angio-associated migratory cell protein.

Figure 3 is a sequence comparison between human sialidases NEU2 and NEU4.

25 **Figure 4** is a table comparing substrate specificity of bacterial and fungal sialidases.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Generally, the nomenclature used herein and the manufacture or laboratory procedures described below are well known and commonly employed in the art. Conventional methods are used for these procedures, such as those provided in the art and various general references. Where a term is provided in the singular, the inventors also contemplate the plural of that term. Where there are discrepancies in terms and definitions used in references that are incorporated by reference, the terms used in this application shall have the definitions given herein. As employed throughout the disclosure, the following terms, unless otherwise indicated, shall be understood to have the following meanings:

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A “pathogen” can be any virus or microorganism that can infect a cell. A pathogen can be a virus, bacterium, or protozoan.

A “target cell” is any cell that can be infected by a pathogen.

An “extracellular activity that can prevent the infection of a target cell by a pathogen” is any activity that can block or impede infection of a target cell by a pathogen by acting at or near the exterior surface of a target cell. The extracellular activity can be an activity such as, but not limited to, a catalytic activity or an inhibitory activity. For example, a catalytic activity can be an enzymatic activity that degrades one or more entities (such as but not limited to ligands, receptors, or enzymes) on a pathogen, on a target cell, or in the vicinity of a target cell, in which the one or more entities contribute to the infection process. A catalytic activity can also modify one or more entities on a pathogen, on a target cell, or in the vicinity of a target cell, such that the infection-promoting property of the entity is reduced. An inhibitory activity can be an activity that, for example, binds to a receptor or ligand and prevents the receptor or ligand from binding a moiety, where the binding is necessary for or promotes the infection process. An inhibitory activity can also be an inhibitor of an enzyme or receptor that prevents the

enzyme or receptor from performing a function that is necessary for or promotes the infection process. The exterior of a target cell includes the target cell membrane itself, as well as the extracellular milieu surrounding the target cell, including extracellular matrix, intracellular spaces, and luminal spaces. For epithelial cells, the exterior of a target cell also includes the apical or luminal surface of the cell membrane that form luminal linings, and the extracellular milieu near the luminal surface. An “extracellular activity that can prevent the infection of a target cell by a pathogen” can be any type of chemical entity, including a protein, polypeptide, peptide, nucleic acid, peptide nucleic acid, nucleic acid analogue, nucleotide, nucleotide analogue, small organic molecule, polymer, lipids, steroid, fatty acid, carbohydrate, and the like, including combinations of any of these. Preferably, however, the activity comprises a peptide or protein or coupled to a peptide or protein.

A “domain that can anchor said at least one therapeutic domain to the membrane of a target cell”, also called an “extracellular anchoring domain” or simply, “anchoring domain” refers to a chemical entity can that can stably bind a moiety that is at or on the exterior of a cell surface or is in close proximity to the surface of a cell. An extracellular anchoring domain can be reversibly or irreversibly linked to one or more moieties, such as, preferably, one or more therapeutic domains, and thereby cause the one or more attached therapeutic moieties to be retained at or in close proximity to the exterior surface of a eukaryotic cell. Preferably, an extracellular anchoring domain binds at least one molecule on the surface of a target cell or at least one molecule found in close association with the surface of a target cell. For example, an extracellular anchoring domain can bind a molecule covalently or noncovalently associated with the cell membrane of a target cell, or can bind a molecule present in the extracellular matrix surrounding a target cell. An extracellular anchoring domain preferably is a peptide, polypeptide, or protein, and can also comprise any additional type of chemical entity, including one or more additional proteins, polypeptides, or peptides, a nucleic acid, peptide nucleic acid, nucleic acid analogue, nucleotide, nucleotide analogue, small organic molecule, polymer, lipids, steroid, fatty acid, carbohydrate, or a combination of any of these.

As used herein, a protein or peptide sequences is “substantially homologous” to a reference sequence when it is either identical to a reference sequence, or comprises one

or more amino acid deletions, one or more additional amino acids, or more one or more conservative amino acid substitutions, and retains the same or essentially the same activity as the reference sequence. Conservative substitutions may be defined as exchanges within one of the following five groups:

- 5 I. Small, aliphatic, nonpolar or slightly polar residues: Ala, Ser, Thr, Pro, Gly
- II. Polar, negatively charged residues and their amides: Asp, Asn, Glu, Gln
- III. Polar, positively charged residues: His, Arg, Lys
- IV. Large, aliphatic nonpolar residues: Met, Leu, Ile, Val, Cys
- 10 V. Large aromatic residues: Phe, Try, Trp

Within the foregoing groups, the following substitution are considered to be "highly conservative": Asp/Glu, His/Arg/Lys, Phe/Tyr/Trp, and Met/Leu/Ile/Val. Semi-conservative substitutions are defined to be exchanges between two of groups (I)-(IV) above which are limited to supergroup (A), comprising (I), (II), and (III) above, or to
15 supergroup (B), comprising (IV) and (V) above. In addition, where hydrophobic amino acids are specified in the application, they refer to the amino acids Ala, Gly, Pro, Met, Leu, Ile, Val, Cys, Phe, and Trp, whereas hydrophilic amino acids refer to Ser, Thr, Asp, Asn, Glu, Gln, His, Arg, Lys, and Tyr.

A "sialidase" is an enzyme that can remove a sialic acid residue from a substrate
20 molecule. The sialidases (N-acetylneuraminosylglycohydrolases, EC 3.2.1.18) are a group of enzymes that hydrolytically remove sialic acid residues from sialo-glycoconjugates. Sialic acids are alpha-keto acids with 9-carbon backbones that are usually found at the outermost positions of the oligosaccharide chains that are attached to glycoproteins and glycolipids. One of the major types of sialic acids is N-acetylneuraminic acid (Neu5Ac),
25 which is the biosynthetic precursor for most of the other types. The substrate molecule can be, as nonlimiting examples, an oligosaccharide, a polysaccharide, a glycoprotein, a ganglioside, or a synthetic molecule. For example, a sialidase can cleave bonds having alpha(2,3)-Gal, alpha(2,6)-Gal, or alpha(2,8)-Gal linkages between a sialic acid residue and the remainder of a substrate molecule. A sialidase can also cleave any or all of the
30 linkages between the sialic acid residue and the remainder of the substrate molecule. Two major linkages between Neu5Ac and the penultimate galactose residues of carbohydrate

side chains are found in nature, Neu5Ac alpha (2,3)-Gal and Neu5Ac alpha (2,6)-Gal. Both Neu5Ac alpha (2,3)-Gal and Neu5Ac alpha (2,6)-Gal molecules can be recognized by influenza viruses as the receptor, although human viruses seem to prefer Neu5Ac alpha (2,6)-Gal, avian and equine viruses predominantly recognize Neu5Ac alpha (2,3)-Gal. A sialidase can be a naturally-occurring sialidase, an engineered sialidase (such as, but not limited to a sialidase whose amino acid sequence is based on the sequence of a naturally-occurring sialidase, including a sequence that is substantially homologous to the sequence of a naturally-occurring sialidase). As used herein, "sialidase" can also mean the active portion of a naturally-occurring sialidase, or a peptide or protein that comprises sequences based on the active portion of a naturally-occurring sialidase.

I. Composition for preventing or treating infection by a pathogen

The present invention includes peptide or protein-based compounds that comprise at least one domain that can anchor at least one therapeutic domain to the membrane of a eukaryotic cell and at least one therapeutic domain having an extracellular activity that can prevent the infection of a cell by a pathogen. By "peptide or protein-based" compounds, it is meant that the two major domains of the compound have an amino acid framework, in which the amino acids are joined by peptide bonds. A peptide or protein-based compound can also have other chemical compounds or groups attached to the amino acid framework or backbone, including moieties that contribute to the anchoring activity of the anchoring domain, or moieties that contribute to the infection-preventing activity or the therapeutic domain. For example, the protein-based therapeutics of the present invention can comprise compounds and molecules such as but not limited to: carbohydrates, fatty acids, lipids, steroids, nucleotides, nucleotide analogues, nucleic acid molecules, nucleic acid analogues, peptide nucleic acid molecules, small organic molecules, or even polymers. The protein-based therapeutics of the present invention can also comprise modified or non-naturally occurring amino acids. Non-amino acid portions of the compounds can serve any purpose, including but not limited to: facilitating the purification of the compound, improving the solubility or distribution or the compound

(such as in a therapeutic formulation), linking domains of the compound or linking chemical moieties to the compound, contributing to the two-dimensional or three-dimensional structure of the compound, increasing the overall size of the compound, increasing the stability of the compound, and contributing to the anchoring activity or therapeutic activity of the compound.

The peptide or protein-based compounds of the present invention can also include protein or peptide sequences in addition to those that comprise anchoring domains or therapeutic domains. The additional protein sequences can serve any purpose, including but not limited to any of the purposes outlined above (facilitating the purification of the compound, improving the solubility or distribution of the compound, linking domains of the compound or linking chemical moieties to the compound, contributing to the two-dimensional or three-dimensional structure of the compound, increasing the overall size of the compound, increasing the stability of the compound, or contributing to the anchoring activity or therapeutic activity of the compound). Preferably any additional protein or amino acid sequences are part of a single polypeptide or protein chain that includes the anchoring domain or domains and therapeutic domain or domains, but any feasible arrangement of protein sequences is within the scope of the present invention.

The anchoring domain and therapeutic domain can be arranged in any appropriate way that allows the compound to bind at or near a target cell membrane such that the therapeutic domain can exhibit an extracellular activity that prevents or impedes infection of the target cell by a pathogen. The compound will preferably have at least one protein or peptide-based anchoring domain and at least one peptide or protein-based therapeutic domain. In this case, the domains can be arranged linearly along the peptide backbone in any order. The anchoring domain can be N-terminal to the therapeutic domain, or can be C-terminal to the therapeutic domain. It is also possible to have one or more therapeutic domains flanked by at least one anchoring domain on each end. Alternatively, one or more anchoring domains can be flanked by at least one therapeutic domain on each end. Chemical, or preferably, peptide, linkers can optionally be used to join some or all of the domains of a compound.

It is also possible to have the domains in a nonlinear, branched arrangement. For example, the therapeutic domain can be attached to a derivatized side chain of an amino

acid that is part of a polypeptide chain that also includes, or is linked to, the anchoring domain.

A compound of the present invention can have more than one anchoring domain. In cases in which a compound has more than one anchoring domain, the anchoring domains can be the same or different. A compound of the present invention can have more than one therapeutic domain. In cases in which a compound has more than one therapeutic domain, the therapeutic domains can be the same or different. Where a compound comprises multiple anchoring domains, the anchoring domains can be arranged in tandem (with or without linkers) or on alternate sides of other domains, such as therapeutic domains. Where a compound comprises multiple therapeutic domains, the therapeutic domains can be arranged in tandem (with or without linkers) or on alternate sides of other domains, such as, but not limited to, anchoring domains.

A peptide or protein-based compound of the present invention can be made by any appropriate way, including purifying naturally occurring proteins, optionally proteolytically cleaving the proteins to obtain the desired functional domains, and conjugating the functional domains to other functional domains. Peptides can also be chemically synthesized, and optionally chemically conjugated to other peptides or chemical moieties. Preferably, however, a peptide or protein-based compound of the present invention is made by engineering a nucleic acid construct to encode at least one anchoring domain and at least one therapeutic domain together (with or without nucleic acid linkers) in a continuous polypeptide. The nucleic acid constructs, preferably having appropriate expression sequences, can be transfected into prokaryotic or eukaryotic cells, and the therapeutic protein-based compound can be expressed by the cells and purified. Any desired chemical moieties can optionally be conjugated to the peptide or protein-based compound after purification. In some cases, cell lines can be chosen for expressing the protein-based therapeutic for their ability to perform desirable post-translational modifications (such as, but not limited to glycosylation).

A great variety of constructs can be designed and their protein products tested for desirable activities (such as, for example, binding activity of an anchoring domain, or a binding, catalytic, or inhibitory activity of a therapeutic domain). The protein products of nucleic acid constructs can also be tested for their efficacy in preventing or impeding

infection of a target cell by a pathogen. *In vitro* and *in vivo* tests for the infectivity of pathogens are known in the art, such as those described in the Examples for the infectivity of influenza virus.

5 *Anchoring Domain*

As used herein, an “extracellular anchoring domain” or “anchoring domain” is any moiety that can stably bind an entity that is at or on the exterior surface of a target cell or is in close proximity to the exterior surface of a target cell. An anchoring domain serves to retain a compound of the present invention at or near the external surface of a
10 target cell.

An extracellular anchoring domain preferably binds 1) a molecule expressed on the surface of a target cell, or a moiety, domain, or epitope of a molecule expressed on the surface of a target cell, 2) a chemical entity attached to a molecule expressed on the surface of a target cell, or 3) a molecule of the extracellular matrix surrounding a target
15 cell.

An anchoring domain is preferably a peptide or protein domain (including a modified or derivatized peptide or protein domain), or comprises a moiety coupled to a peptide or protein. A moiety coupled to a peptide or protein can be any type of molecule that can contribute to the binding of the anchoring domain to an entity at or near the
20 target cell surface, and is preferably an organic molecule, such as, for example, nucleic acid, peptide nucleic acid, nucleic acid analogue, nucleotide, nucleotide analogue, small organic molecule, polymer, lipids, steroid, fatty acid, carbohydrate, or any combination of any of these.

A molecule, complex, domain, or epitope that is bound by an anchoring domain
25 may or may not be specific for the target cell. For example, an anchoring domain may bind an epitope present on molecules on or in close proximity to the target cell and that occur at sites other than the vicinity of the target cell as well. In many cases, however, localized delivery of a therapeutic compound of the present invention will restrict its occurrence primarily to the surface of target cells. In other cases, a molecule, complex,
30 moiety, domain, or epitope bound by an anchoring domain may be specific to a target tissue or target cell type.

Target tissue or target cell type includes the sites in an animal or human body where a pathogen invades or amplifies. For example, a target cell can be an endothelial cell that can be infected by a pathogen. A composition of the present invention can comprise an anchoring domain that can bind a cell surface epitope, for example, that is specific for the endothelial cell type. In another example, a target cell can be an epithelial cell and a composition of the present invention can bind an epitope present on the cell surface of many epithelial cell types, or present in the extracellular matrix of different types of epithelial cells. In this case localized delivery of the composition can restrict its localization to the site of the epithelial cells that are targets of the pathogen.

A compound for preventing or treating infection by a pathogen can comprise an anchoring domain that can bind at or near the surface of epithelial cells. For example, heparan sulfate, closely related to heparin, is a type of glycosaminoglycan (GAG) that is ubiquitously present on cell membranes, including the surface of respiratory epithelium. Many proteins specifically bind to heparin/heparan sulfate, and the GAG-binding sequences in these proteins have been identified (Meyer, FA, King, M and Gelman, RA. (1975) *Biochimica et Biophysica Acta* 392: 223-232; Schauer, S. ed., pp233. Sialic Acids Chemistry, Metabolism and Function. Springer-Verlag, 1982). For example, the GAG-binding sequences of human platelet factor 4 (PF4) (SEQ ID NO:2), human interleukin 8 (IL8) (SEQ ID NO:3), human antithrombin III (AT III) (SEQ ID NO:4), human apoprotein E (ApoE) (SEQ ID NO:5), human angio-associated migratory cell protein (AAMP) (SEQ ID NO:6), or human amphiregulin (SEQ ID NO:7) (Figure 2) have been shown to have very high affinity (in the nanomolar range) towards heparin (Lee, MK and Lander, AD. (1991) *Proc Natl Acad Sci USA* 88:2768-2772; Goger, B, Halden, Y, Rek, A, Mosl, R, Pye, D. Gallagher, J and Kungl, AJ. (2002) *Biochem.* 41:1640-1646; Witt, DP and Lander AD (1994) *Curr Bio* 4:394-400; Weisgraber, KH, Rall, SC, Mahley, RW, Milne, RW and Marcel, Y. (1986) *J Bio Chem* 261:2068-2076). The GAG-binding sequences of these proteins are distinct from their receptor-binding sequences, so they will not induce the biological activities associated with the full-length proteins or the receptor-binding domains. These sequences, or other sequences that have been identified or are identified in the future as heparin/heparan sulfate binding sequences, or sequences substantially homologous to identified heparin/heparan sulfate binding sequences that

have heparin/heparan sulfate binding activity, can be used as epithelium-anchoring-domains in compounds of the present invention that can be used to prevent or treat, for example, respiratory epithelium-infecting viruses such as, but not limited to, influenza virus.

5 An anchoring domain can bind a moiety that is specific to the target cell type of a particular species or can bind a moiety that is found in the target cell type of more than one species. In cases where the anchoring domain can bind moieties that are present at the surface of target cells of more than one species, and a virus or pathogen can infect more than one species, a therapeutic compound can have utility for more than one species
10 (providing that the therapeutic domain is also effective across the relevant species.) For example, in the case of therapeutic compounds that can be used against influenza virus, a therapeutic compound of the present invention that has an anchoring domain that binds heparin/heparan sulfate, the compound can be used in mammals (including humans) as well as avians.

15 *Therapeutic Domain*

 A compound of the present invention includes at least one therapeutic domain that has an extracellular activity that can prevent or impede the infection of a cell by a pathogen. The therapeutic activity can be, as nonlimiting examples, a binding activity, a
20 catalytic activity, or an inhibitory activity. In some embodiments of the present invention, the therapeutic activity acts to modify or inhibit a function of the pathogen that contributes to infectivity of the cell by the pathogen. In other embodiments, a therapeutic domain can modify or inhibit a function of the target cell or target organism.

 For example, the therapeutic domain can bind a receptor on a target cell that is
25 necessary for binding of the pathogen to a target cell. In this way the therapeutic moiety can block binding of the pathogen to a target cell and prevent infection. In an alternative, a therapeutic domain can bind a molecule or epitope on a pathogen to prevent an interaction of the molecule or epitope with a target cell that is necessary for infection. A therapeutic domain can also have a catalytic activity that can degrade a molecule or
30 epitope of the pathogen or host that allows for or promotes infection of a target cell by a host. In yet other embodiments, a therapeutic domain can be an inhibitor of an activity

that is necessary for target cell infection by a pathogen. The inhibited activity can be an activity of the host organism or of the pathogen.

The therapeutic domain preferably acts extracellularly, meaning that its infection-preventing activity takes place at the target cell surface or in the immediate area
5 surrounding the target cell, including sites within the extracellular matrix, intracellular spaces, or luminal spaces of tissues.

A therapeutic domain is preferably a peptide or protein domain (including a modified or derivatized peptide or protein domain), or comprises a moiety coupled to a peptide or protein. A moiety coupled to a peptide or protein can be any type of molecule
10 that can prevent or impede the infection of a target cell by a pathogen, and is preferably an organic molecule, such as, for example, nucleic acid, peptide nucleic acid, nucleic acid analogue, nucleotide, nucleotide analogue, small organic molecule, polymer, lipids, steroid, fatty acid, carbohydrate, or any combination of any of these.

A therapeutic domain can be a synthetic peptide or polypeptide, or can comprise a
15 synthetic molecule that can be conjugated to a peptide or polypeptide, can be a naturally-occurring peptide or protein, or a domain of naturally-occurring protein. A therapeutic domain can also be a peptide or protein that is substantially homologous to a naturally-occurring peptide or protein.

A therapeutic domain can have utility in a particular species, or can prevent or
20 impede pathogen infection in more than one species. For example, therapeutic domains that inhibit pathogen functions can in general be used in a range of species that can be infected by the host, while therapeutic domains that interrupt host-pathogen interactions by interfering with a property of the host may or may not be species-specific. In many cases, anchoring domains and therapeutic domains can be effective in more than one
25 species, so that compounds of the present invention can be used to advance human and animal health, while reducing propagation and spread of the virus through animal hosts. For example, when the therapeutic domain is a sialidase, a sialidase that can cleave more than one type of linkage between a sialic acid residue and the remainder of a substrate molecule, in particular, a sialidase that can cleave both alpha(2, 6)-Gal and alpha (2, 3)-
30 Gal linkages, can protect humans from infections by a broad-spectrum of influenza

viruses, including viruses that are naturally hosted in different species such as birds, pigs or horses.

Linkers

- 5 A compound of the present invention can optionally include one or more linkers that can join domains of the compound. Linkers can be used to provide optimal spacing or folding of the domains of a compound. The domains of a compound joined by linkers can be therapeutic domains, anchoring domains, or any other domains or moieties of the compound that provide additional functions such as enhancing compound stability,
- 10 facilitating purification, etc. A linker used to join domains of compounds of the present invention can be a chemical linker or an amino acid or peptide linker. Where a compound comprises more than one linker, the linkers can be the same or different. Where a compound comprises more than one linker, the linkers can be of the same or different lengths.
- 15 Many chemical linkers of various compositions, polarity, reactivity, length, flexibility, and cleavability are known in the art of organic chemistry. Preferred linkers of the present invention include amino acid or peptide linkers. Peptide linkers are well known in the art. Preferably linkers are between one and one hundred amino acids in length, and more preferably between one and thirty amino acids in length, although
- 20 length is not a limitation in the linkers of the compounds of the present invention. Preferably linkers comprise amino acid sequences that do not interfere with the conformation and activity of peptides or proteins encoded by monomers of the present invention. Some preferred linkers of the present invention are those that include the amino acid glycine. For example, linkers having the sequence:
- 25 (GGGGS (SEQ ID NO:10))_n, where *n* is a whole number between 1 and 20, or more preferably between 1 and 12, can be used to link domains of therapeutic compounds of the present invention.

Composition comprising at least one anchoring domain and at least one protease inhibitor

In some aspects of the present invention, a therapeutic domain that has an extracellular activity that can prevent the infection of a cell by a pathogen is a protease inhibitor. The protease inhibitor can be any type of chemical entity, such as, for example, a carbohydrate or polymer, but is preferably a protein or peptide that inhibits the activity of an enzyme. Preferably, the protease inhibitor inhibits the activity of an enzyme that at least partially processes at least one pathogen or host cell protein, where the processing of the pathogen or host cell protein is necessary for pathogen infectivity. The enzyme that can process a viral protein necessary for pathogen infectivity can be a pathogen enzyme, or an enzyme that originates from the host organism. Preferably, the processing enzyme acts at or near the target cell surface, so that a compound of the present invention that is anchored at or near the surface of a target cell can effectively inhibit the activity of the enzyme.

Compounds of the present invention that comprise protease inhibitory domains can be used to inhibit infection by any pathogen that requires a protease in its life cycle, in which the protease is active at or near the surface of the host cell. These protein-based compositions can have, for example, one of the following structures:

(Anchoring Domain)_n-linker-(Protease Inhibitor)_n (n=1,2, 3 or more)

or :

(Protease Inhibitor)_n-linker-(Anchoring Domain)_n (n=1,2,3 or more)

The protease inhibitor can be a monomeric form of a peptide or polypeptide or can be multiple copies of the same polypeptide that are either linked directly or with spacing sequence in between. Alternatively, different polypeptide-based protease inhibitors can be linked with each other, such as, for example, aprotinin linked with soybean protease inhibitor as protease inhibiting functional domains. The polypeptides or peptides can be linked directly or via a spacer composed of peptide linker sequence. The anchoring domain can be any peptide or polypeptide that can bind at or near the surface of target cells.

The protease inhibitor can be a naturally occurring protease inhibitor (or an active portion thereof) or can be an engineered protease inhibitor. A peptide protease inhibitor used in a compound of the present invention can have a sequence substantially homologous to a naturally occurring protease inhibitor, having one or more deletions, additions, or substitutions while retaining the activity, or substantially retaining the same activity, of the naturally occurring protease inhibitor.

In one preferred embodiment of the present invention, a therapeutic compound of the present invention is for the prevention and treatment of influenza in humans, and the therapeutic domain is a protein or peptide protease inhibitor that can inhibit a serine protease that can cleave the influenza virus hemagglutinin precursor protein HA0 into HA1 and HA2.

A number of serine protease inhibitors have been shown to reduce HA cleavage and influenza virus activation in cultured cells, in chicken embryos and in lungs of infected mice. They include many of the commonly used trypsin inhibitors, such as: aprotinin (Zhirnov OP, Ikizler MR and Wright PF. (2002) *J Virol* 76:8682-8689), leupeptin (Zhirnov OP, Ikizler MR and Wright PF. (2002) *J Virol* 76:8682-8689; Tashiro M, Klenk HD and Rott R.(1987) *J Gen Virol* 68:2039-2043), soybean protease inhibitor (Barbey-Morel CL, Oeltmann TN, Edwards KM and Wright PF. (1987) *J Infect Dis* 155:667-672), e-aminocaproic acid (Zhirnov OP, Ovchartenko AV and Bukrinskaya AG. 1982. *Arch Virol* 73:263-272) and n-p-tosyl-L-lysine chloromethylketone (TLCK) (Barbey-Morel CL, Oeltmann TN, Edwards KM and Wright PF. (1987) *J Infect Dis* 155:667-672). Among these, aerosol inhalation of aprotinin has shown definitive therapeutic effects against influenza and parainfluenza bronchopneumonia in mice (Zhirnov OP, Ovcharenko AV and Bukrinskaya AG. (1984) *J Gen Virol* 65:191-196; Zhirnov OP, Ovcharenko AV and Bukrinskaya AG. (1985) *J Gen Virol* 66:1633-1638; Zhirnov OP. (1987) *J Med Virol* 21:161-167; Ovcharenko AV and Zhirnov OP. (1994) *Antiviral Res* 23:107-118) as well as in human (Zhirnov OP. (1983) *Problems Virol.* 4:9-12 (in Russian)).

Aprotinin (SEQ ID NO: 1; Figure 1) is a 58 amino acid polypeptide inhibitor (also called Trasylol or bovine pancreatic trypsin inhibitor (BPTI)). A compound of the present invention can have one or more aprotinin domains; for example, a therapeutic

composition of the present invention can have from one to six aprotinin polypeptides, more preferably from one to three aprotinin polypeptides. A compound of the present invention can also have a therapeutic domain comprising a polypeptide or peptide having substantial homology to the amino acid sequence of aprotinin.

5 A compound for preventing or treating influenza that comprises a protease inhibitor preferably comprises an anchoring domain that can bind at or near the surface of epithelial cells. In some preferred embodiments, the epithelium anchoring domain is a GAG-binding sequence from a human protein, such as, for example, the GAG-binding sequence of human platelet factor 4 (PF4) (SEQ ID NO:2), human interleukin 8 (IL8)
10 (SEQ ID NO:3), human antithrombin III (AT III) (SEQ ID NO:4), human apoprotein E (ApoE) (SEQ ID NO:5), human angio-associated migratory cell protein (AAMP) (SEQ ID NO:6), or human amphiregulin (SEQ ID NO:7) (Figure 2). A compound of the present invention can also have an anchoring domain comprising a polypeptide or peptide having substantial homology to the amino acid sequences of the GAG-binding domains
15 listed in SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, and SEQ ID NO:7.

Clinically, a drug comprising aprotinin and an epithelial anchoring domain can be administered by aerosol inhalation to cover the entire respiratory tract to prevent and treat bronchopneumonia caused by influenza viruses, or any other virus, such as parainfluenza
20 virus, that requires serine proteases in its life cycle. Alternatively, an aprotinin/epithelial anchoring domain fusion protein can be administered as nasal spray to treat uncomplicated early stage influenza cases or other infections by respiratory viruses. In addition, an aprotinin/epithelial anchoring domain fusion protein can be used as a prophylaxis for influenza or other viral infections before an infection occurs.

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Composition comprising at least one anchoring domain and at least one catalytic activity

In some aspects of the present invention, a therapeutic domain that has an extracellular activity that can prevent the infection of a cell by a pathogen is a catalytic activity. The enzymatic activity can be a catalytic activity that removes, degrades or
30 modifies a host molecule or complex or a pathogen molecule or complex that contributes to the infectivity of the pathogen. Preferably the host molecule or complex or pathogen

molecule or complex that is removed, degraded, or modified by the enzymatic activity of a compound of the present invention is on, at, or near the surface of a target cell, so that a compound of the present invention that is anchored to the surface of a target cell can effectively inhibit the host or pathogen molecule or complex.

5 For example, a therapeutic domain can have a catalytic activity that can digest a molecule or epitope of the pathogen or target cell that is required for host-pathogen binding, and subsequent entry of the pathogen into the target cell. Receptors on target cells that allow for the entry of viruses into cells can be the target of an enzymatic activity of a compound of the present invention.

10 Compounds of the present invention that comprise catalytic domains can be used to inhibit infection by any pathogen that uses a receptor to gain entry to a target cell, as long as removal of the receptor does not impair the organism. These protein-based compositions can have, for example, one of the following structures:

15 (Anchoring Domain)_n-[linker]-(Enzymatic Activity)_n (n=1,2, 3 or more)

 or :

 (Enzymatic Activity)_n (n=1,2, 3 or more)-[linker]-(Anchoring Domain)_n,

 where the linkers are optional.

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 The enzymatic activity can be a monomeric form of a peptide or polypeptide or can be multiple copies of the same polypeptide that are either linked directly or with spacing sequence in between. The polypeptides or peptides can be linked directly or via a spacer composed of peptide linker sequence. The anchoring domain can be any peptide or polypeptide that can bind to or near the surface of target cells.

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 In one preferred embodiment of the present invention, a therapeutic domain comprises a sialidase that can eliminate or greatly reduce the level of sialic acid on the surface of epithelial cells. Sialic acid is a receptor for influenza viruses. Thus, treating the surface of respiratory epithelial cells with a sialidase can prevent influenza infections or interrupt early infections. The therapeutic domain can comprise a complete sialidase protein, or an active portion thereof. Compositions can be tested for their efficacy in

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cleaving sialic acid residues and reducing infection of target cells by influenza virus or other pathogens using methods known in the art and described in the examples.

Preferred sialidases are the large bacterial sialidases that can degrade the receptor sialic acids Neu5Ac alpha(2,6)-Gal and Neu5Ac alpha(2,3)-Gal. For example, the
5 bacterial sialidase enzymes from *Clostridium perfringens* (Genbank Accession Number X87369), *Actinomyces viscosus* (Genbank Accession Number X62276), *Arthrobacter ureafaciens*, or *Micromonospora viridifaciens* (Genbank Accession Number D01045) can be used. Therapeutic domains of compounds of the present invention can comprise all or a portion of the amino acid sequence of a large bacterial sialidase or can comprise amino
10 acid sequences that are substantially homologous to all or a portion of the amino acid sequence of a large bacterial sialidase. Other preferred sialidases are the human sialidases such as those encoded by the genes NEU2 (SEQ ID NO:8; Genbank Accession Number Y16535; Monti, E, Preti, Rossi, E., Ballabio, A and Borsani G. (1999) *Genomics* 57:137-143) and NEU4 (SEQ ID NO:9; Genbank Accession Number NM080741; Monti, E,
15 Preti, A, Venerando, B and Borsani, G. (2002) *Neurochem Res* 27:646-663) (Figure 3). Therapeutic domains of compounds of the present invention can comprise all or a portion of the amino acid sequences of a human sialidase or can comprise amino acid sequences that are substantially homologous to all or a portion of the amino acid sequences of a human sialidase. Preferably, where a therapeutic domain comprises a portion of the
20 amino acid sequences of a naturally occurring sialidase, or sequences substantially homologous to a portion of the amino acid sequences of a naturally occurring sialidase, the portion comprises essentially the same activity as the human sialidase.

A compound for preventing or treating influenza that comprises an enzymatic domain preferably comprises an anchoring domain that can bind at or near the surface of
25 epithelial cells. In some preferred embodiments, the epithelium-anchoring domain is a GAG-binding sequence from a human protein, such as, for example, the GAG-binding amino acid sequences of human platelet factor 4 (PF4) (SEQ ID NO:2), human interleukin 8 (IL8) (SEQ ID NO:3), human antithrombin III (AT III) (SEQ ID NO:4), human apoprotein E (ApoE) (SEQ ID NO:5), human angio-associated migratory cell
30 protein (AAMP) (SEQ ID NO:6), and human amphiregulin (SEQ ID NO:7) (Figure 2).

An epithelial anchoring domain can also be substantially homologous to a naturally occurring GAG-binding sequence, such as those listed in Figure 2.

It is also within the scope of the present invention to use compounds comprising a human sialidase, or comprising a sialidase with substantial homology to a human sialidase, in the absence of an anchoring domain, in the treatment or prevention of pathogen infections, such as but not limited to influenza, paramyxovirus, coronavirus, rotavirus, and *Pseudomonas aeruginosa* infections. The present invention recognizes that such infections may be prevented or abated by the use of sialidases, such as, but not limited to, human sialidases such as NEU2 and NEU4. The sialidases can optionally be adapted, by genetic or chemical engineering, or by pharmaceutical formulation, to improve their half life or retention at the respiratory epithelium.

Because influenza viruses primarily infect the upper respiratory tract, removing the receptor sialic acid locally in the nasal cavity and nasopharynx area can prevent infections or interrupt early infections. The sialidase can be delivered to the upper respiratory tract as a nasal spray, and it can be used either in therapeutic mode during early stage of influenza (or other infection) or in prophylactic mode before the infection occurs. Alternatively, it can be delivered to the lower respiratory tract as an inhalant to treat influenza and to prevent influenza complications, such as bronchopneumonia.

II. Pharmaceutical Compositions

The present invention includes compounds of the present invention formulated as pharmaceutical compositions. The pharmaceutical compositions comprise a pharmaceutically acceptable carrier prepared for storage and preferably subsequent administration, which have a pharmaceutically effective amount of the compound in a pharmaceutically acceptable carrier or diluent. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, 18th Ed., Mack Publishing Co., Easton, PA (1990)). Preservatives, stabilizers, dyes and even flavoring agents can be provided in the

pharmaceutical composition. For example, sodium benzoate, sorbic acid and esters of p-hydroxybenzoic acid can be added as preservatives. In addition, antioxidants and suspending agents can be used.

Depending on the target cell, the compounds of the present invention can be formulated and used as tablets, capsules or elixirs for oral administration; salves or ointments for topical application; suppositories for rectal administration; sterile solutions, suspensions, and the like for use as inhalants or nasal sprays. Injectables can also be prepared in conventional forms either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. Suitable excipients are, for example, water, saline, dextrose, mannitol, lactose, lecithin, albumin, sodium glutamate, cysteine hydrochloride and the like. In addition, if desired, the injectable pharmaceutical compositions can contain minor amounts of nontoxic auxiliary substances, such as wetting agents, pH buffering agents and the like.

The pharmaceutically effective amount of a test compound required as a dose will depend on the route of administration, the type of animal or patient being treated, and the physical characteristics of the specific animal under consideration. The dose can be tailored to achieve a desired effect, but will depend on such factors as weight, diet, concurrent medication and other factors which those skilled in the medical arts will recognize. In practicing the methods of the present invention, the pharmaceutical compositions can be used alone or in combination with one another, or in combination with other therapeutic or diagnostic agents. These products can be utilized *in vivo*, preferably in a mammalian patient, preferably in a human, or *in vitro*. In employing them *in vivo*, the pharmaceutical compositions can be administered to the patient in a variety of ways, including topically, parenterally, intravenously, subcutaneously, intramuscularly, colonically, rectally, nasally or intraperitoneally, employing a variety of dosage forms. Such methods can also be used in testing the activity of test compounds *in vivo*.

In preferred embodiments, these pharmaceutical compositions may be in the form of orally-administrable suspensions, tablets; nasal sprays; or inhalants.

When administered orally as a suspension, compositions of the present invention are prepared according to techniques well-known in the art of pharmaceutical formulation and may contain microcrystalline cellulose for imparting bulk, alginic acid or sodium alginate as a suspending agent, methylcellulose as a viscosity enhancer, and sweeteners/flavoring agents known in the art. As immediate release tablets, these compositions may contain microcrystalline cellulose, dicalcium phosphate, starch, magnesium stearate and lactose and/or other excipients, binders, extenders, disintegrants, diluents and lubricants known in the art. Components in the formulation of a mouthwash or rinse include antimicrobials, surfactants, cosurfactants, oils, water and other additives such as sweeteners/flavoring agents known in the art.

When administered by a drinking solution, the composition comprises one or more of the compounds of the present invention, dissolved in water, with appropriate pH adjustment, and with carrier. The compound may be dissolved in distilled water, tap water, spring water, and the like. The pH can preferably be adjusted to between about 3.5 and about 8.5. Sweeteners may be added, e.g., 1% (w/v) sucrose.

Lozenges can be prepared according to U.S. Patent No. 3,439,089, herein incorporated by reference for these purposes.

When administered by nasal aerosol or inhalation, the pharmaceutical compositions are prepared according to techniques well-known in the art of pharmaceutical formulation and may be prepared as solutions in saline, employing benzyl alcohol or other suitable preservatives, absorption promoters to enhance bioavailability, fluorocarbons, and/or other solubilizing or dispersing agents known in the art. See, for example, Ansel, H. C. et al., *Pharmaceutical Dosage Forms and Drug Delivery Systems*, Sixth Ed. (1995). Preferably these compositions and formulations are prepared with suitable nontoxic pharmaceutically acceptable ingredients. These ingredients are known to those skilled in the preparation of nasal dosage forms and some of these can be found in Remington's *Pharmaceutical Sciences*, 18th Ed., Mack Publishing Co., Easton, PA (1990, a standard reference in the field. The choice of suitable carriers is

highly dependent upon the exact nature of the nasal dosage form desired, e.g., solutions, suspensions, ointments, or gels. Nasal dosage forms generally contain large amounts of water in addition to the active ingredient. Minor amounts of other ingredients such as pH adjusters, emulsifiers or dispersing agents, 5 preservatives, surfactants, jelling agents, or buffering and other stabilizing and solubilizing agents may also be present. Preferably, the nasal dosage form should be isotonic with nasal secretions.

Nasal formulations can be administered as drops, sprays, aerosols or by any other intranasal dosage form. Optionally, the delivery system can be a unit dose 10 delivery system. The volume of solution or suspension delivered per dose can preferably be anywhere from about 5 to about 2000 microliters, more preferably from about 10 to about 1000 microliters, and yet more preferably from about 50 to about 500 microliters. Delivery systems for these various dosage forms can be dropper bottles, plastic squeeze units, atomizers, nebulizers or pharmaceutical 15 aerosols in either unit dose or multiple dose packages.

The formulations of this invention may be varied to include; (1) other acids and bases to adjust the pH; (2) other tonicity imparting agents such as sorbitol, glycerin and dextrose; (3) other antimicrobial preservatives such as other parahydroxy benzoic acid esters, sorbate, benzoate, propionate, chlorbutanol, 20 phenylethyl alcohol, benzalkonium chloride, and mercurials; (4) other viscosity imparting agents such as sodium carboxymethylcellulose, microcrystalline cellulose, polyvinylpyrrolidone, polyvinyl alcohol and other gums; (5) suitable absorption enhancers; (6) stabilizing agents such as antioxidants, like bisulfite and ascorbate, metal chelating agents such as sodium edetate and drug solubility 25 enhancers such as polyethylene glycols.

III. Method of preventing or treating infection by a pathogen

The present invention also includes methods of preventing or treating infection by a pathogen. The method includes: treating a subject that is infected with a pathogen or at risk of being infected with a pathogen with a pharmaceutical composition of the present invention that comprises a compound that comprises at least one anchoring domain that can anchor the compound at or near the surface of a target cell and at least one therapeutic domain comprising a peptide or protein that has at least one extracellular activity that can prevent the infection of a target cell by a pathogen. The subject to be treated can be an animal or human subject.

Compounds of the present invention can be designed for human use or animal use. In some aspects of the present invention, a compound of the present invention can be used to prevent pathogen infection in a class of animals, such as mammals. In some aspects of the present invention, a composition can be used for human and animal use (although the formulation may differ). In these aspects, the active domains of a compound can be effective against more than one pathogen species, type, subtype, or strain and can be active in more than one host species. For example, some preferred compounds of the present invention that comprise, for example, active domains such as protease inhibitors that prevent processing of the HA protein of influenza virus, or sialidases that remove sialic acid receptors from target cells, or anchoring domains such as domains that bind heparin or heparan sulfate, can be used in birds, mammals, or humans. Such compounds that can be effective against a range of pathogens with the capacity to infect different host species can also be used in humans to combat infection by pathogens that are naturally hosted in other species.

In some preferred embodiments of the present invention, the pharmaceutical composition prevents infection by influenza, and a therapeutically effective amount of the pharmaceutical composition is applied to the respiratory epithelial cells of a subject. This can be done by the use of an inhaler, or by the use of a nasal spray. Preferably, the inhaler or nasal spray is used from one to four times a day.

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Dosage

As will be readily apparent to one skilled in the art, the useful *in vivo* dosage to be administered and the particular mode of administration will vary depending upon the age, weight and type of patient being treated, the particular pharmaceutical composition employed, and the specific use for which the pharmaceutical composition is employed. The determination of effective dosage levels, that is the dose levels necessary to achieve the desired result, can be accomplished by one skilled in the art using routine methods as discussed above. In non-human animal studies, applications of the pharmaceutical compositions are commenced at higher dose levels, with the dosage being decreased until the desired effect is no longer achieved or adverse side effects are reduced or disappear. The dosage for a compound of the present invention can range broadly depending upon the desired affects, the therapeutic indication, route of administration and purity and activity of the compound. Typically, human clinical applications of products are commenced at lower dosage levels, with dosage level being increased until the desired effect is achieved. Alternatively, acceptable *in vitro* studies can be used to establish useful doses and routes of administration of the test compound. Typically, dosages can be between about 1 ng/kg and about 10 mg/kg, preferably between about 10 ng/kg and about 1 mg/kg, and more preferably between about 100 ng/kg and about 100 micrograms/kg.

The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition (see, Fingle et al., in *The Pharmacological Basis of Therapeutics* (1975)). It should be noted that the attending physician would know how to and when to terminate, interrupt or adjust administration due to toxicity, organ dysfunction or other adverse effects. Conversely, the attending physician would also know to adjust treatment to higher levels if the clinical response were not adequate. The magnitude of an administered dose in the management of the disorder of interest will vary with the severity of the condition to be treated and to the route of administration. The severity of the condition may, for example, be evaluated, in part, by standard prognostic evaluation methods. Further, the dose and perhaps dose frequency,

will also vary according to the age, body weight and response of the individual patient, including those for veterinary applications.

Thus, in accordance with the present invention, there is further provided a method of treating and a pharmaceutical composition for treating influenza virus infection and prevention of influenza virus infection. The treatment involves administering to a patient in need of such treatment a pharmaceutical carrier and a therapeutically effective amount of any composition of the present invention, or a pharmaceutically acceptable salt thereof.

In one preferred regimen, appropriate dosages are administered to each patient by either nasal spray or by oral lozenge. It will be understood, however, that the specific dose level and frequency of dosage for any particular patient may be varied and will depend upon a variety of factors including the activity of the specific salt or other form employed, the metabolic stability and length of action of that compound, the age, body weight, general health, sex, diet, mode and time of administration, rate of excretion, drug combination, the severity of the particular condition, and the host undergoing therapy.

Examples

Example 1: Synthesizing aprotinin genes, purifying and testing aprotinin fusion proteins.

Introduction

5 Influenza viral protein hemagglutinin (HA) is the major influenza envelope protein. It plays an essential role in viral infection. The importance of HA is evidenced by the fact that it is the major target for protective neutralizing antibodies produced by the host immune response (Hayden, FG. (1996) In *Antiviral drug resistance* (ed. D. D. Richman), pp. 59-77. Chichester, UK: John Wiley & Sons Ltd.). It is now clear that HA
10 has two different functions in viral infection. First, HA is responsible for the attachment of the virus to sialic acid cell receptors. Second, HA mediates viral entry into target cells by triggering fusion of the viral envelope with cellular membranes.

 HA is synthesized as a precursor protein, HA0, which is transferred through the Golgi apparatus to the cell surface as a trimeric molecular complex. HA0 is further
15 cleaved to generate the C terminus HA1 (residue 328 of HA0) and the N terminus of HA2. It is generally believed that the cleavage occurs at the cell surface or on released viruses. The cleavage of HA0 into HA1/HA2 is not required for HA binding to a sialic acid receptor; however, it is essential for viral infectivity (Klenk, HD and Rott, R. (1988) *Adv Vir Res.* 34:247-281; Kido, H, Niwa, Y, Beppu, Y and Towatari, T. (1996) *Advan*
20 *Enzyme Regul* 36:325-347; Skehel, JJ and Wiley, DC. (2000) *Annu Rev Biochem* 69:531-569).

 Sensitivity of HA0 to host proteases is determined by the proteolytic site in the external loop of HA0 molecule. The proteolytic site may contain either a single Arg or Lys residue (monobasic cleavage site) or several Lys and/or Arg residues in R-X-K/R-R
25 motif (multibasic cleavage site). Only the influenza A virus subtypes H5 and H7 have HA proteins carrying the multibasic cleavage site. All other influenza A, B and C viruses contain HA proteins having the monobasic cleavage site. Influenza A viruses having multibasic cleavage sites are more virulent and induce systemic infection in hosts
 whereas viruses with a monobasic HA site initiate infection only in the respiratory tract in
30 mammals or in the respiratory and enteric tracts in avian species (Klenk, HD and Garten W. 1994. *Trend Micro* 2:39-43 for review). Fortunately, human infection by the highly

virulent avian influenza A H5 and H7 subtypes, which carry the multibasic cleavage site, has so far only occurred in a handful of cases discovered mostly in Hong Kong. The vast majority of influenza infections are caused by viruses with HA proteins are cleaved at the monobasic cleavage site.

5 Influenza virus HA subtypes 5 and 7 that contain multibasic cleavage sites are activated by furin, a member of the subtilisin-like endoproteases, or the pre-protein convertase family. Furin cleaves the virus intracellularly and is ubiquitously present in many cell types, allowing the virulent, systemic infection seen with such viruses (Klenk, HD and Garten W. 1994. *Trend Micro* 2:39-43; Nakayama, K. 1997. *Biochem* 327:625-10 635). All other influenza viruses, which have HAs with monobasic cleavage sites, are activated by secreted, trypsin-like serine proteases. Enzymes that have been implicated in influenza virus activation include: plasmin (Lazarowitz SG, Goldberg AR and Choppin PW. 1973. *Virology* 56:172-180), mini-plasmin (Murakami M, Towatari T, Ohuchi M, Shiota M, Akao M, Okumura Y, Parry MA and Kido H. (2001) *Eur J Biochem* 268:15 2847-2855), tryptase Clara (Kido H, Chen Y and Murakami M. (1999) In B.Dunn (ed.), *Proteases of infectious agents*. p.205-217, Academic Press, New York, N.Y), kallikrein, urokinase, thrombin (Scheiblaue H, Reinacher M, Tashiro M and Rott R. (1992) *J Infect Dis* 166:783-791), blood clotting factor Xa (Gotoh B, Ogasawara T, Toyoda T, Inocencio N, Hamaguchi M and Nagai Y. (1990) *EMBO J* 9:4189-4195), acrosin (Garten W, Bosch20 FX, Linder D, Rott R and Klenk HD. (1981) *Virology* 115:361-374.), proteases from human respiratory lavage (Barbey-Morel CL, Oeltmann TN, Edwards KM and Wright PF. (1987) *J Infect Dis* 155:667-672) and bacterial proteases from *Staphylococcus aureus* (Tashiro M, Ciborowski P, Reinacher M, Pulverer G, Klenk HD and Rott R. (1987) *Virology* 157:421-430) and *Pseudomonas aeruginosa* (Callan RJ, Hartmann FA,25 West SE and Hinshaw VS. (1997) *J Virol* 71:7579-7585). Activation of influenza viruses by host serine proteases is generally considered to occur extracellularly either at the plasma membrane or after virus release from the cell.

 Aprotinin, also called Trasylol, or bovine pancreatic trypsin inhibitor (BPTI) is a polypeptide having 58 amino acids. It belongs to the family of Kunitz-type inhibitors and30 competitively inhibits a wide spectrum of serine proteases, including trypsin, chymotrypsin, plasmin and plasma kallikrein. Aprotinin has long been used as a human

therapeutics, such as treatment of pancreatitis, various states of shock syndrome, hyperfibrinolytic haemorrhage and myocardial infarction. It is also used in open-heart surgery, including cardiopulmonary bypass operations, to reduce blood loss (Fritz H and Wunderer G. (1983) *Arzneim-Forsch* 33:479-494).

5 The safety of aprotinin in human has been well documented through years of clinical applications. In addition, aprotinin is apparently a very weak immunogen as aprotinin-specific antibodies have not been observed in human sera so far (Fritz H and Wunderer G. (1983) *Arzneim-Forsch* 33:479-494). Another desired feature of aprotinin as a drug candidate is its superb stability. It can be kept at room temperature for at least
10 18 months without any loss of activity (Fritz H and Wunderer G. (1983) *Arzneim-Forsch* 33:479-494).

 To achieve significant viral inhibition in animal studies that have been performed, aprotinin was administered at high doses. For example, 280 micrograms to 840 micrograms per day of aprotinin was injected intraperitoneally into each mouse for 6
15 days (Zhirnov OP, Ovcharenko AV and Bukrinskaya AG. (1984) *J Gen Virol* 65:191-196); a lower dosage was required for aerosol inhalation, still, each mouse was given 63 - 126 micrograms per day for 6 days (Ovcharenko AV and Zhirnov OP. (1994) *Antiviral Res* 23:107-118). A very high dose of aprotinin would be required in human based on extrapolation from the mouse data. Therefore to achieve better efficacy in human, the
20 potency of aprotinin molecule needs to be significantly improved.

 Aprotinin functions by competitively inhibiting serine proteases that are mostly on the surface of host respiratory epithelial cells. Local concentration of aprotinin in the vicinity of host proteases is therefore the key factor determining competitive advantage of aprotinin. We use two approaches that work synergistically to boost competitive
25 advantage of aprotinin on the surface of respiratory epithelium.

 First, the avidity (functional affinity) of aprotinin is increased by making multivalent aprotinin fusion proteins consisting of two, three, or more aprotinin proteins connected via linkers. Such a molecule is able to bind to membrane proteases in a multivalent fashion, which has significant kinetic advantage over the aprotinin monomer.
30 Monomeric aprotinin binds to bovine trypsin very tightly with dissociation constant (K_i) being 6.0×10^{-14} mol/l. However, its affinity compared to other proteases, such as

chymotrypsin, plasmin and Kallikrein, which have been implicated in activation of influenza viruses, is much lower with K_i being at the level of 10^{-8} to 10^{-9} mol/l (Fritz H and Wunderer G. (1983) *Arzneim-Forsch* 33:479-494). Multimerization can increase aprotinin's affinity to these proteases exponentially.

5 Second, we fuse aprotinin with a respiratory epithelium-anchoring domain. The anchoring domain localizes aprotinin to the proximity of host membrane-associated proteases and maintains a high local concentration of aprotinin on epithelial surface. The anchoring domain also increases retention time of the drug on the respiratory epithelium.

10 Cloning

Aprotinin is a single chain polypeptide having 58 amino acid residues and 3 intra-chain disulfide bonds (SEQ ID NO:1). The amino acid sequence of aprotinin is shown in Figure 1. Genes encoding aprotinin and aprotinin fusion proteins are synthesized by PCR using overlapping oligonucleotides with codons optimized for *E. Coli* expression as
15 templates. The PCR products are cloned into pCR2.1-TOPO vector (Invitrogen). After sequencing, the genes are subcloned into an expression vector pQE (Qiagen). The vector carries a purification tag, His₆, to allow easy purification of the recombinant proteins. The constructs are used to transform *E. Coli*. The transformed cells grown in LB-ampicillin medium to mid-log phase are induced by IPTG according to standard
20 protocols. Cells are pelleted and lysed in phosphate-buffered-saline (PBS) by sonication. The enzymes, which have His₆ purification tag, are purified using a nickel column (Qiagen).

The following aprotinin fusion proteins are made:

1. Dimeric and trimeric aprotinin. Two or three aprotinin genes are linked via a flexible
25 linker as the following constructs:

Aprotinin—(GGGGS (SEQ ID NO:10))_n (n=3, 4 or 5)—Aprotinin;

and

Aprotinin—(GGGGS(SEQ ID NO:10))_n (n=3, 4 or 5)—Aprotinin—

30 (GGGGS(SEQ ID NO:10))_n (n=3, 4 or 5)—Aprotinin

The length of the linker sequence may determine three-dimensional flexibility of the multimeric aprotinin and thereby influence functional affinity of the molecule. Therefore constructs having linkers with various lengths are made.

Fully functional recombinant monomeric aprotinin has been produced in *E. Coli* (Auerswald EA, Horlein D, Reinhardt G, Schroder W and Schnabel E. (1988). *Biol Chem Hoppe-Seyler* Vol 369, Suppl., pp27-35). We therefore expect proper folding of multivalent aprotinin proteins in *E. coli* cells. Besides expressing protein in various common *E. Coli* cell strains, such as BL21, JM83, etc, the multivalent aprotinin proteins are also expressed in OrigamiTM cells (Novagen, Bad Soden, Germany). The OrigamiTM cell strain does not have thioredoxin and glutathione reductase and thus has an oxidizing cytoplasm. This cell strain has been used to successfully express a number of proteins that contain disulfide bonds (Bessette PH, Aslund F, Beckwith J and Georgiou G. (1999) *Pro Natl Acad Sci USA* 96:13703-13708; Venturi M, Seifert C and Hunte C. (2001) *J Mol Biol* 315:1-8.).

2. The epithelium cell-anchoring aprotinin. An epithelium cell-anchoring sequence is fused with aprotinin. The epithelium-anchoring sequence can be any peptide or polypeptide sequence that has affinity towards the surface of epithelial cells. We have selected three human GAG-binding sequences: PF4 (aa 47-70; SEQ ID NO: 2), IL-8 (aa 46-72; SEQ ID NO: 3), and AT III (aa 118-151; SEQ ID NO: 4) (Figure 2). These sequences bind to heparin/heparan sulfate with nanomolar-level affinities (Table 1). Heparin/Heparan Sulfate are ubiquitously present on the respiratory epithelium. In separate constructs, the GAG-binding sequences are fused with the aprotinin gene on the N terminus and on the C terminus via a generic linker sequence GGGGS as the following constructs:

(GAG domain—GGGGS(SEQ ID NO:10)—Aprotinin); and
(Aprotinin—GGGGS(SEQ ID NO:10)—GAG domain)

Table 1. Affinities to Heparin

5	Protein	Kd nM (ref)
	PF4	27 (44)
	IL-8	<5 (43)
	ATIII	11 (42)
10	ApoE	620 (45)

Photometric trypsin inhibition assay

15 The trypsin inhibition activity of aprotinin and aprotinin fusion proteins is measured by a photometric assay described previously in detail (Fritz H and Wunderer G. (1983) *Arzneim-Forsch* 33:479-494). Briefly, in this assay aprotinin inhibits the trypsin-catalyzed hydrolysis of Na-benzoyl-L-arginine-p-nitroanilide (BzArgpNA or L-BAPA) (Sigma), which is followed photometrically at 405 nm. One trypsin unit (U_{BAPA})

20 corresponds to the hydrolysis of 1 micromole substrate per min. One inhibitor unit (IU_{BAPA}) decreases the activity of two trypsin units by 50%, which corresponds arithmetically to the inhibition of 1 U_{BAPA} of trypsin. The specific activity of aprotinin is given in IU_{BAPA}/mg polypeptide.

25 *Surface plasmon resonance assay*

 The affinities of dimeric and trimeric aprotinin with various linkers are compared against the monomeric aprotinin using surface plasmon resonance assay, or BIAcore analysis (BIAcore, Piscataway, NJ) with human plasmin as the target. Similarly, BIAcore assay with heparin as the target is used to analyze affinity between GAG

30 binding aprotinin fusion proteins and heparin.

When plasmin is used as the target, purified human plasmin (Sigma) is immobilized on the CM5 chip according manufacturer's instructions (BIAcore, Piscataway, NJ). When heparin is the target, biotinylated albumin and albumin-heparin (Sigma) are captured on a streptavidin-coated BIAcore SA chip as described previously
5 (Xiang Y and Moss B. (2003) *J Virol* 77:2623-2630).

Example 2: Establishing improved tissue culture models for studies on influenza virus infection.

Stocks of Influenza Viruses

10 Influenza viral strains are obtained from ATCC and the repository at St. Jude Children's Research Hospital. All experiments involving influenza viruses are conducted at Bio-safety level II.

Viruses are propagated by injection into the allantoic cavity of nine-day-old chicken embryos as described (Zhirnov OP, Ovcharenko AV and Bukrinskaya AG.
15 (1985) *J Gen Virol* 66:1633-1638). Alternatively, viral stocks are grown on Madin-Darby canine kidney (MDCK) cells in minimal essential medium (MEM) supplemented with 0.3% bovine serum albumin and 0.5 micrograms of trypsin per ml. After incubating for 48 to 72 hours, the culture medium is clarified by low speed centrifugation. Viral particles are pelleted by ultracentrifugation through a 25% sucrose
20 cushion. Purified viruses are suspended in 50% glycerol-0.1M Tris buffer (pH 7.3) and stored at -20°C .

Plaque Assays

Infectivity and titer of the viral stocks are determined by two kinds of plaque
25 assays, a conventional one and a modified one (Tobita, K, Sugiura, A, Enomoto, C and Furuyama, M. (1975) *Med Microbiol Immunol* 162:9-14; Zhirnov OP, Ovcharenko AV and Bukrinskaya AG. (1982) *Arch Virol* 71:177-183). The conventional plaque assay is routinely used as a virus titration method. It requires exogenous trypsin in agar overlay added immediately after virus infection to MDCK monolayers (Tobita, K, Sugiura, A,

Enomoto, C and Furuyama, M. (1975) *Med Microbiol Immunol* 162:9-14). This method artificially increases infectivity of the viral stocks being tested by activating all the viral particles that have uncleaved HA.

Zhirnov et. al. designed a modified plaque assay consisting of a double agar overlay, with trypsin being included in the second layer which is added 24 hours after infection (Zhirnov OP, Ovcharenko AV and Bukrinskaya AG. (1982) *Arch Virol* 71:177-183). Three days after infection, cells are fixed with a 10% formaldehyde solution, agarose layers are removed, fixed cells are stained with hematoxylin-eosin solution and plaques are counted. The modified plaque assay allows accurate determination of the real infectivity of viral stocks that contain both cleaved and uncleaved HA. Combining results from both conventional and modified plaque assays, one can distinguish viruses containing cleaved or uncleaved HA and correlate infectivity of viral stocks with the status of HA cleavage.

15 *Human Cell Culture Models*

1. Short-term culture of primary human epithelial cells. Conventional *in vitro* influenza virus infection is mostly carried out in MDCK cells with exogenous trypsin added to the culture medium. This is far from being physiological and is inappropriate for the work proposed here because trypsin is not the protease that activate influenza viruses *in vivo*. Very limited numbers of *in vitro* tissue culture models that are able to support the growth of influenza virus without an exogenous protease have been reported so far, those being primary cultures with primate cells of renal origin, cells lining the allantoic and amniotic cavities of embryonated eggs, fetal tracheal ring organ cultures and primary human adenoid epithelial cells (Endo Y, Carroll KN, Ikizler MR and Wright PF. (1996) *J Virol* 70:2055-2058). Among these, the latest work with primary human adenoid epithelial cells is the closest mimic of human conditions. In this case, Endo et. al. (Endo Y, Carroll KN, Ikizler MR and Wright PF. (1996) *J Virol* 70:2055-2058) isolated epithelial cells from surgical samples of human adenoids, and cultured the epithelial cells on a collagen matrix (Vitrogen 100, Celtrix Laboratories, Palo Alto, California) in Transwell inserts (Costar, Cambridge, Mass). Cells were maintained in 50% Ham's F12 and 50% Eagles

minimal essential media with supplements of growth factors and trace elements. The cells reached confluency in 10 to 14 days, remaining largely as a monolayer but with discrete patches of ciliated cells, which maintained regular ciliary activity for 1 to 3 weeks after reaching confluency. In this system, influenza A virus grew to a titer of 10^6 PFU/ml with a multiplicity of infection of 0.001 (Endo Y, Carroll KN, Ikizler MR and Wright PF. (1996) *J Virol* 70:2055-2058). Progressive cytopathogenic effects were also present during infection. The biggest drawback of this system is that it requires fresh human adenoid tissue.

To solve this problem, primary human adenoid epithelial cells are replaced with primary human airway epithelial cells that are commercially available (Cambrex), and the cells are grown under the same conditions. Such short-term culture of primary human airway epithelial cells is relatively quick to establish and is useful as the first-line experimental model for most of the *in vitro* infection and antiviral experiments.

2. Well-differentiated human airway epithelium (WD-HAE). In order to best mimic the *in vivo* condition of human airway, the model of well-differentiated human airway epithelium (WD-HAE) is used. WD-HAE is stratified epithelium that has all the differentiated cells of the normal human airway epithelium, including functional ciliated cells and mucus secreting cells. Therefore, in this model system influenza viruses are most likely to be activated by host proteases that are physiologically relevant. Although WD-HAE has been widely used to study respiratory viral infections, such as respiratory syncytial virus (RSV) Zhang L, Peeples ME, Boucher RC, Collins PL and Pickles RJ. (2002) *J Virol* 76:5654-5666) measles virus (Sinn PL, Williams G, Vongpunsawad S, Cattaneo R and McCray PB. (2002) *J Virol* 76:2403-2409, or human rhinovirus, it has not previously been used to study influenza viruses.

A detailed protocol of WD-HAE has been described previously (Krunkosky TM, Fischer BM, Martin LD, Jones N, Akley NJ and Adler KB. (2000) *Am J Respir Cell Mol Biol* 22:685-692). Briefly, commercial primary human bronchial epithelial cells (Cambrex) are cultured on Transwell-clear culture inserts (Costar) that are thin-coated with rat-tail collagen I. Cells are cultured submerged for the first 5 to 7 days in medium containing a 1:1 mixture of bronchial epithelial cell growth medium (BEGM) (Cambrex)

and DMEM with high glucose with supplement of growth factors (Krunkosky TM, Fischer BM, Martin LD, Jones N, Akley NJ and Adler KB. (2000) *Am J Respir Cell Mol Biol* 22:685-692). When cultures are 70% confluent (days 5 to 7), the air-liquid interface is created by removing the apical medium and exposing cells only to medium on their basal surface. Cells are cultured for additional 14 days in air-liquid interphase, for a total of 21 days in culture, and are then ready for experiments. The differentiated epithelium can be maintained *in vitro* for weeks.

Epithelial morphology and degree of differentiation is documented by routine histology (Endo Y, Carroll KN, Ikizler MR and Wright PF. (1996) *J Virol* 70:2055-2058). Briefly, following fixation with 10% buffered formalin, the epithelial cells are embedded in paraffin, sectioned and stained with hematoxylin and eosin, and with periodic acid-Schiff stain for mucus secreting cells.

Influenza infection is carried out in the above two model systems by adding 0.001 to 1 MOI of viruses to the differentiated cells. The titer and infectivity of viruses in the supernatant are followed over a period of 3 to 7 days. The level of influenza viral amplification and the infectivity of influenza viruses are evaluated using conventional and modified plaque assays.

Example 3: Comparing functions of the aprotinin fusion proteins in vitro

Anti-Viral Effects of Aprotinin Fusion Proteins

1. Pre-infection treatment. Aprotinin fusion proteins are added to primary human cell cultures at various concentrations and allowed to incubate with the cells for 1 hour. The cells are washed with fresh medium and immediately inoculated with influenza viruses at MOI 0.01 to 1. Cells are washed again after 1 hour and cultured for 3 to 5 days. Titer and infectivity of viruses in the supernatant are measured at various time points by two plaque assays. The cytopathic effect caused by viral infection is evaluated by staining viable cells with crystal violet and quantifying by measuring absorption at 570 nm at the end of the experiment. The percentage of cell protection by aprotinin fusion proteins is calculated by $100 \times \{(\text{aprotinin treated sample} - \text{untreated infected sample}) / (\text{uninfected control} - \text{untreated infected sample})\}$. The drug efficacy for cell

protection is described by its Effective Concentration that achieves 50% of the cell protection (EC_{50}). Since HA activation only occurs to newly released viral particles, the first round of viral infection occurs normally and viral titer rises in the first 24 hours after infection. However, starting from the second round, infectivity of viruses drops and viral titer gradually decreases as result of aprotinin treatment. Results from this experiment differentiate various types of different aprotinin fusion proteins by their efficacies in a single prophylactic treatment.

Alternatively, timing of initial viral inoculation is altered from immediately after aprotinin treatment to 2-24 hours post treatment. Viral titer, infectivity and cytopathic effect are measured for 3 to 5 day after infection as described above. Results from these experiments distinguish various aprotinin fusion proteins by the lengths of the effective window after a single prophylactic treatment.

2. Post-infection Treatment. For multi-dose treatment, cells are first infected by viral inoculations at 0.001 to 0.1 MOI for 1 hour. Various concentrations of aprotinin fusion proteins are added immediately afterwards, additional treatments are applied at 8-hour intervals during the first 48 hours post infection. Cells are cultured until day 7 post infection. Viral titer and infectivity in the media are followed during the whole process. Cytopathic effect is evaluated at the end of the experiment.

For single dose treatment, cells are first infected by viral inoculations at 0.001 to 0.1 MOI for 1 hour. Treatments of aprotinin fusion proteins at various concentrations are applied at different time points during the first 48 hours after infection, but each cell sample only receives one treatment during the whole experiment. Cells are cultured until day 7 post infection. Viral titer and infectivity in the media are followed during the whole process. Cytopathic effect is evaluated at the end of the experiment. Results from these experiments distinguish different types of aprotinin fusion proteins for their therapeutic potency.

Inhibition of HA Cleavage by Aprotinin Fusion Proteins

To demonstrate that aprotinin fusion proteins inhibit influenza viral infection by inhibiting cleavage of influenza HA protein, a human primary epithelial cell culture is infected with influenza virus at MOI of 1. Aprotinin fusion proteins are added to the culture either right before viral inoculation or immediately after the viral infection. At 6.5 hour post infection, the culture is incubated for 1 hour in MEM lacking cold methionine and containing ³⁵S-labeled methionine (Amersham) at a concentration of 100 microCi/ml (pulse). Thereafter, the cells are washed twice with MEM containing a 10-fold concentration of cold methionine and incubated in MEM for additional 3 hours (chase). After labeling, cells are dissolved in radioimmunoprecipitation assay (RIPA) buffer, HA is precipitated by anti-serum against the particular strain of virus used for infection (anti-influenza sera can be obtained from ATCC and Center of Disease Control and Prevention), and immunocomplex is then purified by protein G-Sepharose (Amersham). Samples are fractionated by SDS-PAGE followed by autoradiography. In samples untreated by aprotinin fusion proteins, HA1 and HA2 are expected to be the predominant HA species; while in aprotinin treated samples, HA0 is expected to be the major type of HA present.

Example 4: Synthesizing genes of five sialidases, expressing and purifying the sialidase proteins.

Introduction

Influenza viruses belong to the *orthomyxoviridae* family of RNA viruses. Both type A and type B viruses have 8 segmented negative-strand RNA genomes enclosed in a lipid envelope derived from the host cell. The viral envelope is covered with spikes that are composed of three proteins: hemagglutinin (HA), that attaches virus to host cell receptors and mediates fusion of viral and cellular membranes; neuraminidase (NA), which facilitates the release of the new viruses from the host cell; and a small number of M2 proteins that serve as ion channels. For *Influenza A virus*, HA and NA both undergo antigenic drift and antigenic shift, the viral subtypes are distinguished by serologic differences between their HA and NA proteins. There are total 15 types of HA (H1-H15)

and 9 types of NA (N1-N9), but only three HA (H1-H3) and two NA (N1 and N2) have been found in human *Influenza A virus* so far (Granoff, A. & Webster, R. G., ed. *Encyclopedia of Virology*, 2nd Edition, Vol 2). In contrast to *Influenza A virus*, no distinct antigenic subtypes are recognized for *Influenza virus B*.

5 While *Influenza B virus* circulates only in humans, *Influenza A virus* can be isolated from a whole host of animals, such as pigs, horses, chickens, ducks and other kinds of birds, which accounts for genetic reassortment of *Influenza A virus* that results in antigenic shift. Wild aquatic birds are considered to be the primordial reservoir of all influenza viruses for avian and mammalian species. There is extensive evidence for
10 transmission of the virus between aquatic birds and other species including pigs and horses and indirect transmission to humans through pigs. Direct transmission from pigs or chickens to humans has also been documented (Ito, T. (2000) *Microbiol Immunol* 44(6):423-430).

 The host cell receptor for influenza viruses is the cell surface sialic acid. Sialic
15 acids are α -keto acids with 9-carbon backbones that are usually found at the outermost positions of the oligosaccharide chains that are attached to glycoproteins and glycolipids. One of the major types of sialic acids is N-acetylneuraminic acid (Neu5Ac), which is the biosynthetic precursor for most of the other types. Two major linkages between Neu5Ac and the penultimate galactose residues of carbohydrate side chains are found in nature,
20 Neu5Ac α (2,3)-Gal and Neu5Ac α (2,6)-Gal. Both Neu5Ac α (2,3)-Gal and Neu5Ac α (2,6)-Gal molecules can be recognized by *Influenza A virus* as the receptor (Schauer, R. (1982) *Adv. Carbohydrate Chem & Biochem* 40:131-235), while human viruses seem to prefer Neu5Ac α (2,6)-Gal, avian and equine viruses predominantly recognize Neu5Ac α (2,3)-Gal (Ito, T. (2000) *Microbiol Immunol* 44(6):423-430).

25 Infections by influenza type A and B viruses are typically initiated at the mucosal surface of the upper respiratory tract. Viral replication is primarily limited to the upper respiratory tract but can extend to the lower respiratory tract and causes bronchopneumonia that can be fatal. The risk of death is one per 10,000 infections, but is significantly greater for high-risk groups with pre-existing cardiopulmonary conditions
30 and for immunologically naïve individuals during a pandemic.

A therapeutic compound comprising a sialidase that can effectively degrade both receptor sialic acids, Neu5Ac α (2,6)-Gal and Neu5Ac α (2,3)-Gal, can confer protection against the broadest range of influenza viruses, including animal viruses. It can also remain effective as the viral strains change yearly. Because sialidase targets the host cell rather than virus and acts at the “choking point” in a viral life cycle, generation of resistant virus is improbable. Protein-bound sialic acid turns over homogeneously on cell surface with half-life of 33 hours (Kreisel, W, Volk, BA, Buchsel, R. and Reutter, W. (1980) *Proc Natl Acad Sci USA* 77:1828-1831). Therefore we estimate that once-a-day or twice-a-day administration of a sialidase would confer sufficient protection against influenza.

Sialidases are found in higher eukaryotes, as well as in some mostly pathogenic microbes, including viruses, bacteria and protozoans. Viral and bacterial sialidases have been well characterized, and the three-dimensional structures of some of them have been determined (Crennell, SJ, Garman, E, Laver, G, Vimr, E and Taylor, G. (1994) *Structure* 2:535-544; Janakiraman, MN, White, CL, Laver, WG, Air, GM and Luo, M. (1994) *Biochemistry* 33:8172-8179; Pshezhetsky, A, Richard, C, Michaud, L, Igdoura, S, Wang, S, Elsliger, M, Qu, J, Leclerc, D, Gravel, R, Dallaire, L and Potier, M. (1997) *Nature Genet* 15: 316-320). Several human sialidases have also been cloned in the recent years (Milner, CM, Smith, SV, Carrillo MB, Taylor, GL, Hollinshead, M and Campbell, RD. (1997) *J Bio Chem* 272:4549-4558; Monti, E, Preti, A, Nesti, C, Ballabio, A and Borsani G. 1999. *Glycobiol* 9:1313-1321; Wada, T, Yoshikawa, Y, Tokuyama, S, Kuwabara, M, Akita, H and Miyagi, T. (1999) *Biochem Biophys Res Communi* 261:21-27; Monti, E, Bassi, MT, Papini, N, Riboni, M, Manzoni, M, Veneranodo, B, Croci, G, Preti, A, Ballabio, A, Tettamanti, G and Borsani, G. (2000) *Biochem J* 349:343-351). All the sialidases characterized share a four amino acid motif in the amino terminal portion followed by the Asp box motif which is repeated three to five times depending on the protein. (Monti, E, Bassi, MT, Papini, N, Riboni, M, Manzoni, M, Veneranodo, B, Croci, G, Preti, A, Ballabio, A, Tettamanti, G and Borsani, G. (2000) *Biochem J* 349:343-351; Copley, RR, Russell, RB and Ponting, CP. (2001) *Protein Sci* 10:285-292). While the overall amino acid identity of the sialidase superfamily is relatively low at about 20-30%, the overall fold of the molecules, especially the catalytic amino acids, are remarkably

similar (Wada, T, Yoshikawa, Y, Tokuyama, S, Kuwabara, M, Akita, H and Miyagi, T. (1999) *Biochem Biophys Res Commun* 261:21-27; Monti, E, Bassi, MT, Papini, N, Riboni, M, Manzoni, M, Veneranodo, B, Croci, G, Preti, A, Ballabio, A, Tettamanti, G and Borsani, G. (2000) *Biochem J* 349:343-351; Copley, RR, Russell, RB and Ponting, CP. (2001) *Protein Sci* 10:285-292).

The sialidases are generally divided into two families: “small” sialidases have molecular weight of about 42 kDa and do not require divalent metal ion for maximal activity; “large” sialidases have molecular weight above 65 kDa and may require divalent metal ion for activity (Wada, T, Yoshikawa, Y, Tokuyama, S, Kuwabara, M, Akita, H and Miyagi, T. (1999) *Biochem Biophys Res Commun* 261:21-27; Monti, E, Bassi, MT, Papini, N, Riboni, M, Manzoni, M, Veneranodo, B, Croci, G, Preti, A, Ballabio, A, Tettamanti, G and Borsani, G. (2000) *Biochem J* 349:343-351; Copley, RR, Russell, RB and Ponting, CP. (2001) *Protein Sci* 10:285-292).

Over fifteen sialidase proteins have been purified and they vary greatly from one another in substrate specificities and enzymatic kinetics. To confer a broad-spectrum protection against influenza viruses, a sialidase needs to effectively degrade sialic acid in both $\alpha(2,6)$ -Gal and $\alpha(2,3)$ -Gal linkages and in the context of glycoproteins and some glycolipids. Viral sialidases, such as those from *influenza A virus*, *fowl plague virus* and *Newcastle disease virus*, are generally specific for Neu5Ac $\alpha(2,3)$ -Gal and only degrade Neu5Ac $\alpha(2,6)$ -Gal very inefficiently. Small bacterial sialidases generally react poorly to sialic acid in the context of glycoproteins and glycolipids. By contrast, large bacterial sialidases can effectively cleave sialic acid in both ($\alpha,2-6$) linkage and ($\alpha,2-3$) linkage in the context of most natural substrates (Figure 4; Vimr, DR. (1994) *Trends Microbiol* 2: 271-277; Drzeniek, R. (1973) *Histochem J* 5:271-290; Roggentin, P, Kleineidam, RG and Schauer, R. (1995) *Biol Chem Hoppe-Seyler* 376:569-575; Roggentin, P, Schauer, R, Hoyer, LL and Vimr, ER. (1993) *Mol Microb* 9:915-921). Because of their broad substrate specificities, large bacterial sialidases are better candidates.

Among the large bacterial sialidases with known substrate specificity shown in Figure 4, *Vibrio cholerae* sialidase requires Ca^{2+} for activity making it less preferred. More preferred sialidases include the 71 kDa enzyme from *Clostridium perfringens*, the 113 kDa enzyme from *Actinomyces viscosus* and sialidase of *Arthrobacter ureafaciens*.

A third sialidase, the 68 kDa enzyme from *Micromonospora viridifaciens*, has been known to destroy influenza viral receptor (Air, GM and Laver, WG. (1995) *Virology* 211:278-284), and is also a candidate.

These enzymes have high specific activity (600 U/mg protein for *C. perfringens* (Corfield, AP, Veh, RW, Wember, M, Michalski, JC and Schauer, R. (1981) *Biochem J* 197:293-299) and 680 U/mg protein for *A. viscosus* (Teufel, M, Roggentin, P. and Schauer, R. (1989) *Biol Chem Hoppe Seyler* 370:435-443)), are fully active without divalent metal iron, and have been cloned and purified as recombinant proteins from *E. coli* (Roggentin, P, Kleineidam, RG and Schauer, R. (1995) *Biol Chem Hoppe-Seyler* 376:569-575, Teufel, M, Roggentin, P. and Schauer, R. (1989) *Biol Chem Hoppe Seyler* 370:435-443 , Sakurada, K, Ohta, T and Hasegawa, M. (1992) *J Bacteriol* 174: 6896-6903). In addition, *C. perfringens* is stable in solution at 2-8°C for several weeks, and at 4°C in the presence of albumin for more than two years (Wang, FZ, Akula, SM, Pramod, NP, Zeng, L and Chandran, B. (2001) *J Virol* 75:7517-27). *A. viscosus* is labile towards freezing and thawing, but is stable at 4°C in 0.1 M acetate buffer, pH 5 (Teufel, M, Roggentin, P. and Schauer, R. (1989) *Biol Chem Hoppe Seyler* 370:435-443).

Although the chances of inducing immune reactions using bacterial sialidases is very low because the proteins will be used topically in the upper respiratory tract and will not be absorbed systemically a human enzyme would be more desirable for long-term use in human subjects.

Four sialidase genes have been cloned from human so far: NEU1/G9/lysosomal sialidase (Pshezhetsky, A, Richard, C, Michaud, L, Igdoura, S, Wang, S, Elsliger, M, Qu, J, Leclerc, D, Gravel, R, Dallaire, L and Potier, M. (1997) *Nature Genet* 15: 316-320. , Milner, CM, Smith, SV, Carrillo MB, Taylor, GL, Hollinshead, M and Campbell, RD. (1997). *J Bio Chem* 272:4549-4558); NEU3, a membrane-associated sialidase isolated from human brain (Wada, T, Yoshikawa, Y, Tokuyama, S, Kuwabara, M, Akita, H and Miyagi, T. (1999) *Biochem Biophys Res Communi* 261:21-27, Monti, E, Bassi, MT, Papini, N, Riboni, M, Manzoni, M, Veneranodo, B, Croci, G, Preti, A, Ballabio, A, Tettamanti, G and Borsani, G. (2000) *Biochem J* 349:343-351), NEU2 a 42 kDa sialidase expressed in human skeletal muscle at a very low level (Monti, E, Preti, A, Nesti, C, Ballabio, A and Borsani G. (1999) *Glycobiol* 9:1313-1321), and NEU4 a 497 amino acid

protein (Genebank NM080741) expressed in all human tissues examined (Monti, E, Preti, A, Venerando, B and Borsani, G. (2002) *Neurochem Res* 27:646-663).

Amino acid sequence comparison reveals NEU2 (SEQ ID NO:8) and NEU4 (SEQ ID NO:9) are both cytosolic sialidases. 9 out of 12 of the amino acid residues which form the catalytic site of *S. typhimurium* sialidase are conserved in both NEU2 and NEU4 (Monti, E, Preti, A, Nesti, C, Ballabio, A and Borsani G. (1999) *Glycobiol* 9:1313-1321, Figure 3). In addition, NEU4 also shows a stretch of about 80 amino acid residues (aa 294-373) that appears unique among known mammalian sialidases (Monti, E, Preti, A, Venerando, B and Borsani, G. (2002) *Neurochem Res* 27:646-663). Unlike the selected large bacterial sialidases, the substrate specificity of NEU2 and NEU4 is unknown. It will need to be tested if NEU2 and NEU4 can effectively degrade the influenza virus receptors.

Sialidase assay

NEU2, NEU4 and *M. viridifaciens* enzymes will be stored in PBS and 50% glycerol at -20°C . *C. perfringens* and *A. viscosus* enzymes are stored in 10mM acetate buffer (pH5) at 4°C . Protein preps are characterized by HPLC and SDS-PAGE electrophoresis. Specific activities and stability of the enzymes will be monitored by sialidase assay.

The enzymatic activity of sialidases are determined by fluorimetric 2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid (4MU-NANA) (Sigma) as the substrate. Specifically, reactions are set up in duplicate in 0.1M Na citrate/phosphate buffer pH5.6, in the presence of 400 micrograms bovine serum albumin, with 0.2 mM 4MU-NANA in a final volume of 100 microliters, and incubated at 37°C for 5-10min. Reactions are stopped by addition of 1 ml of 0.2 M glycines/NaOH pH10.2. Fluorescence emission is measured on a fluorometer with excitation at 365 nm and emission at 445 nm, using 4-methylumbelliferone (4-MU) to obtain a calibration curve.

Example 5: Comparing functions of the sialidases in vitro and selecting one sialidase for further studies.

1. Stocks of Influenza Viruses

Influenza viral strains are obtained from the ATCC and the repository at St. Jude Children's Research Hospital. Viral stocks are grown on Madin-Darby canine kidney (MDCK) cells in minimal essential medium (MEM) supplemented with 0.3% bovine serum albumin and 0.5 micrograms of trypsin per ml. After incubating for 48 to 72 hours, the culture medium is clarified by low speed centrifugation. Viral particles are pelleted by ultracentrifugation through a 25% sucrose cushion. Purified viruses are suspended in 50% glycerol-0.1M Tris buffer (pH 7.3) and stored at -20°C . Viral titer is determined by plaque assay (Tobita, K, Sugiura, A, Enomoto, C and Furuyama, M. (1975) *Med Microbiol Immunol* 162: 9-14), or TCID_{50} , which is the dose of virus required to infect 50% of the MDCK cells.

Selected human and animal influenza A strains with specificity towards Neu5Ac alpha(2,6)-Gal or Neu5Ac alpha(2,3)-Gal and have high affinity to the receptors (measured by high hemagglutination activity) are chosen for *in vitro* tests:

1. Strains that recognize receptor Neu5Ac alpha(2,6)-Gal include human isolates A/aichi/2/68, A/Udorn/307/72, A/Prot Chaimers/1/73 and A/Victoria/3/75, etc. (Connor, RJ, Kawaoka, Y, Webster, RG and Paulson JC. (1994) *Virology* 205:17-23).

2. Strains that have Neu5Ac alpha(2,3)-Gal specificity include animal isolates A/duckUkraine/1/63, A/duckMemphis/928/74, A/duckhokk/5/77, A/Eq/Miami/1/63, A/Eq/Ur/1/63, A/Eq/Tokyo/71, A/Eq/Prague/71, etc (Connor, RJ, Kawaoka, Y, Webster, RG and Paulson JC. (1994) *Virology* 205:17-23).

2. Hemagglutination Assay

This assay is used to rapidly determine the efficiency of each enzyme to destroy receptors Neu5Ac alpha(2,6)-Gal and Neu5Ac alpha(2,3)-Gal.

Specifically, 6 ml of Chicken red blood cells (SPAFAS Inc., Norwich, CT) are diluted in two times the volume of PBS, centrifuge for 5 min at 500 x g and re-suspended in PBS of original volume. Sialidases are added to the chicken erythrocytes at various concentrations and allowed to incubate at room temperature for 30 min. The cells are then washed three times to remove sialidase proteins, and then are resuspended in PBS to 6 ml. Control cells are incubated with BSA and washed. Various strains of influenza virus, which recognize either Neu5Ac alpha(2,6)-Gal or Neu5Ac salpha(2,3)-Gal as the receptor as listed above, are prepared in microtiter plates as serial dilutions in PBS (100 microliters) of the original viral stocks. Sialidase-treated or control chicken red blood cell suspensions (100 microliters of the 0.5% solution prepared above) are added to each well at 4°C. The plates are read after 2 h. The lowest concentration of virus that causes the blood cell to agglutinate is defined as one hemagglutination unit. We will be looking for enzymes that effectively abolish hemagglutination by all viral strains.

3. Viral Inhibition Assay

Confluent monolayers of MDCK cells are treated with various concentrations of sialidases for 1 h, washed twice with buffer, then infected with various strains of influenza virus. After incubation for 1 hr, the cells are washed again to remove unbound virus. To estimate the decrease in viral binding sites on cell surface, the cells are overlaid with agar and incubated at 37°C. The number of plaques in the sialidase treated cells will be compared against those in control cells. Alternatively, the cells will be cultured in regular medium at 37°C, and viral titers in the culture media are measured at various time during culture as TCID₅₀.

To demonstrate that sialidase treatment can inhibit a pre-existing infection, MDCK monolayers are first infected with a low titer of virus. After washing off the unbound virus, the cells are then cultured in the presence of a sialidase. Fresh sialidase is added to cell culture every 24 h. Viral titer in the cultured medium is measured over a 72-hour period.

4. Cytotoxicity assay

Primary human bronchial epithelial cells are purchased (Clonetics) and cultured in supplemented minimal medium following manufacture's instruction. Sialidases are added to the culture medium at various concentrations. Cell growth over a period of 7-10 days will be measured. Cells will also be observed regularly for microscopic cytopathic effects.

Example 6: Constructing and testing sialidase fusion proteins.

1. Choosing a GAG-binding sequence as the anchoring domain.

One sialidase is selected for its best overall properties, including anti-viral activity, toxicity, stability, ease of production, etc. We will then genetically link it to a GAG-binding sequence, sub-clone the fusion genes into pQE vector, express and purify the fusion proteins from *E. coli*.

We have selected six possible human GAG-binding sequences: PF4 (aa 47-70) (SEQ ID NO:2), IL-8 (aa 46-72) (SEQ ID NO:3), AT III (aa 118-151) (SEQ ID NO:4), ApoE (aa 132-165) (SEQ ID NO:5), amphiregulin (aa 25-45) (SEQ ID NO:6), and human angio-associated migratory cell protein (AAMP) (aa 14-25) (SEQ ID NO:7) (Figure 2). These sequences generally bind to heparin with nanomolar-level affinities; however, their affinities may vary from one another by an order of magnitude (Table 1). Since it is not clear which anchoring domain will enable the most effective functioning of the sialidase, all four GAG-binding sequences are fused with the sialidase gene either on the N terminus or the C terminus via a generic linker sequence GGGGS as the following constructs:

(GAG binding domain— GGGGS(SEQ ID NO:10) —Sialidase); or

(Sialidase—GGGGS(SEQ ID NO:10)—GAG binding domain)

Different fusion proteins are compared by a modified viral inhibition assay. Specifically, confluent monolayers of MDCK cells are treated with same amount of each fusion protein for a limited duration, such as 30 min. The cells are then washed twice with buffer to remove unbound sialidase fusion proteins, and incubated in culture medium for an additional 1 hour. Afterwards, strains of influenza virus are added to the cells for 1 hr and then cells are washed again to remove unbound virus. Viral titers in the culture media are measured during 72-h cultures as TCID₅₀. The un-fused sialidase protein will be used to compare against the fusion proteins in this assay. If the results are too close to rank all fusion proteins, we will make the assay more stringent by shortening treatment window for the fusion proteins, lowering protein concentrations and increasing the level of viral challenge.

2. Optimizing the fusion protein construct

After selecting the best fusion protein from the earlier experiments, the construct is further optimized by testing different linker length. In this regard, the following constructs are made:

(Sialidase—(GGGGS(SEQ ID NO:10))_n (n=0, 1, 2, 3, or 4) —GAG binding domain)

The proteins are expressed and purified and compared in the modified viral protection assay as described above.

In addition, if earlier data indicate that higher affinity of the fusion protein towards heparan sulfate brings better potency, we also plan to test if the potency can be further improved by increasing the GAG-binding affinity. This can be achieved by creating a multivalent GAG binding mechanism in the fusion protein in constructs like these:

(Sialidase—(GGGGS(SEQ ID NO:10))_n—HS binding domain—GAG binding domain);

or:

(GAG binding domain—(GGGGS(SEQ ID NO:10))_n—Sialidase—

(GGGGS(SEQ ID NO:10))_n—GAG binding domain)

The purified fusion proteins are ranked based on their activities in the modified viral protection assay as described above.

3. Cytotoxicity assay

- 5 The effects of the fusion proteins on normal cell growth and morphology are monitored by culturing primary human bronchial epithelial cells with various concentrations of the fusion proteins and following growth curve of the cells and observing any microscopic cytopathic effects.

10 ***Example 7: Fusion Proteins against Other Infectious Microbes***

- Fusion proteins composed of a functional domain and an anchorage domain are designed for many more different applications. For example, a sialidase fusion protein as proposed here can also be used as a therapeutic/prophylactic agent against infections by other viruses and bacteria besides influenza viruses, because many other infectious
- 15 microbes, such as paramyxoviruses (Wassilewa, L. (1977) *Arch Virol* 54:299-305), coronaviruses (Vlasak, R., Luytjes, W., Spaan, W. and Palese, P. (1988) *Proc Natl Acad Sci USA* 85:4526-4529), rotaviruses (Fukudome, K., Yoshie, O. and Konno, T. (1989) *Virology* 172:196-205) and *Pseudomonas aeruginosa* (Ramphal, R. and Pyle, M. (1983) *Infect Immun* 41:339-44) etc, are also known to use sialic acid as cellular receptors. For
- 20 example, aprotinin fused with a heparin-binding domain can make a fusion protein that be used to prevent/treat infection of other viruses besides influenza that require host serine proteases from activation, such as parainfluenza virus.

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- 25 All publications, including patent documents, Genbank sequence database entries including nucleotide and amino acid sequences and accompanying information, and scientific articles, referred to in this application and the bibliography and attachments are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication were individually incorporated by reference.

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